PLK1 controls centriole distal appendage formation and centrobin removal via independent pathways

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AUTHORS: Morgan LeRoux-Bourdieu, Daniela Harry, and Patrick Meraldi
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We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This is an interesting article on the dynamics of CNTROB and its role in distal appendages in human cell lines.
It includes new data, which in general terms appears to be solid and reaches some interesting conclusions.
These results merit publication once the following important points are addressed.

Comments for the author

1.- One main concern is about the major conclusion: the higher affinity of CNTRB for the newly born daughter centrioles is the cause of CNTRB removal from older centrioles. The authors themselves seem to be aware that this conclusion rests on thin evidence when in the introduction they write: “possibly because of higher affinity binding sites”. “Possibly” is the correct term here, but it is not used anywhere else in the manuscript. For instance in the abstract: “centrobin is removed from older centrioles “due to a higher affinity for the newly born daughter centrioles”; and in one heading of the results section: “Centrobin is removed from mother centriole due to a higher affinity for daughter centrioles”.
It is fairly obvious that proving this claim will take a great deal more than a series of immunofluorescence data.
The provided data is fairly consistent with the authors’s interpretation, but does not falsify alternative hypotheses e.g daughter centrioles act catalytically. The authors must either provide additional data that demonstrate their conclusion or re-phrase the manuscript accordingly.
2.- In Figure 2, CNTROB signal intensity is much lower in the mother centriole of the PLK4i-treated cell than in the daughter centriole of the control cell. This result is not consistent with the hypothetical mechanism proposed in the manuscript. If CNTROB removal was due to a higher affinity for daughter centrioles CNTROB signal intensity should be as high in PLK4i-treated than in control cells. Quantitated data on CNTROB signal intensity in these centrioles are in order.
3.- The point that CNTROB localisation is compatible with OFD1 and CEP164 is important, but Figure 3B,E does not serve the purpose because it does not show CNTROB. Bearing in mind Figure 2 one can guess the result, but co-staining for CNTROB is a must to confirm its presence on these centrioles. This is even more important when taking into account Wang et al., 2018 showing that PACT-CNTROB, which forces CNTROB centriolar localisation through the cell cycle, results in the loss of appendages.
4.- Another major issue that requires attention derives from drawing general conclusions out of specific observations. Results from the only one animal in which Centrobin has been studied in vivo demonstrate that Centrobin is a multifaceted protein that can exert different, sometimes seemingly contradictory functions.
Thus, for instance Centrobin promotes PCM recruitment during interphase in Drosophila neuroblasts, but not in other cell types like epithelial wing disc cells. Likewise Centrobin inhibits centriole-to-basal body conversion in type I neurons (Gottardo et al., 2015 DOI: 10.1016/j.cub.2015.07.038) but is required for proper basal body function in spermatocytes (Reina et al., 2018 DOI: 10.1083/jcb.201801032). Incidentally Centrobin's function inhibiting cilium growth in Drosophila neurons could be mentioned in the manuscript.
Statements assuming that observations made on a handful of human cell lines can be generalized to “mammalian cells” are likely to be proven wrong once in vivo studies allow for Centrobin dynamics and function to be studied in different cell types in a living mammalian experimental model. In Drosophila, as explained before, statements generalising Centrobin function are definitively wrong and so are general statements on Centrobin localisation which is daughter centriole-specific in many somatic cells and early stage germline cells, but localises to both mother and daughter centrioles in primary spermatocytes (Reina et al., 2018).

Throughout the manuscript, conclusions should refer to the cell type in which the observation has been made hence avoiding unwarranted generalisations. This applies both to the new data reported in the manuscript as well as to references to published data.
5.-Being so heavily based on immunofluorescence, it would be advisable to back up at least the conclusions derived from Figure 1 with a second anti-CNTROB antibody (if I am not mistaken, the entire manuscript relies on the Abcam anti-CNTROB).
6.- There are recurrent mistakes regarding the description of Centrobin function and localisation in Drosophila that must be amended:
6a.- "In Drosophila neuroblasts, which contain appendage-free centrioles, centrobin is exclusively present on the younger centrosome containing the mother centriole, whose identity it controls (Januschke et al., 2013)"
6b.-"note that in flies centrobin is present not on daughter centrioles but on the younger centrosome (Januschke et al., 2013)"
6c.- "In Drosophila, centrobin localization has to be controlled since it regulates the fate of the young centrosome", enabling it to organize microtubules and to be retained by the stem cell during asymmetric cell division (Januschke et al., 2013)."
The authors got Centrobin localisation in Drosophila neuroblasts wrong. In these cells, CNB is only present in the DAUGHTER centriole, not in the mother. And the daughter centriole is in the younger centrosome.
Once more, general statements like "note that in flies" or "In Drosophila" are bound to be wrong, and so are these ones. Centrobin is daughter centriole-specific in neuroblasts and male germline stem cells, for instance, but it is not daughter centriole-specific in primary spermatocytes. Moreover, Centrobin effect on the young centrosome's fate is only true in neuroblasts. It is not true in other Drosophila cells including stem cells like those of the male germline.

Other points The results regarding the effect of Cenexin, centriolin and CEP128 depletion in mitosis are clear, but what happens to CNTROB localisation in G1?

Figure 4h, Aurora-Ai: the two middle panels are swapped.
Figure 7b: Is in unclear which panels belong to each genotype
The article demonstrating that in Drosophila neuroblasts Centrobin is daughter centriole-bound and that these cells retain the younger (daughter) centrosome is Januschke et al., 2011
https://doi.org/10.1038/ncomms1245

Reviewer 2

Advance summary and potential significance to field

Centrosomes are organelles built of centrioles, cylindrical MT-based structures that, when fully assembled carry subdistal and distal appendages, responsible for MT anchoring, and ciliation, respectively. Centrobin is a centrosomal protein enriched to the sites of developing centrioles from their earliest stages. During centriole development, centrobin levels are reduced. This reduction has been suggested to be a prerequisite for the formation of centriole appendages. In this work, Le Roux-Bourdieu and colleagues explore the timing and the consequences of centrobin removal from mother centrioles, and the role of Plk1 and subdistal appendage proteins in this process.

They find that centrobin is lost from younger mother centrioles during their second mitosis. They show that distal appendage proteins re recruited to mother centrioles independently of centrobin removal. They further demonstrate that Plk1 controls centrobin localization at centrosomes, but independently from distal appendage proteins. Finally, using knockouts for subdistal appendage proteins cenexin/Odf2, Centriolin and Cep128, they show that removal of these proteins leads to retention of some centrobin on mother centrioles.

There are interesting findings in the manuscript. For instance, the timeline of centrobin removal is described in more detail than before. They clarify that localization of Cep164 does not require removal of centrobin from mother centrioles. The authors show that Plk1 activity can modulate centrosomal levels of centrobin. However, there are issues with data interpretation, proposed concepts, and image quality. Nevertheless, the subject is important and interesting, and the study could be publishable after an extensive revision.

Comments for the author

Point 1. Immunofluorescence signals are hard to analyze because they are distorted. Specifically, they are elongated diagonally, always in same direction, which indicates some systemic issue with imaging. Because of that, it remains unclear how accurate are the quantifications of centrosome phenotypes and centrobin IF signals. At a minimum, the data in 1D, 2A, 2D, 3B, 5G, 7B, and 7F needs to be replaced with properly acquired, high-quality images.
Point 2. Per quantification in (Fig. 1A), centrobin is associated with both centrioles in ~10% of G1 cells. However, the number of cells with both mother centrioles associating with centrobin increases in S and G2 to 15% and >25% respectively. Have some older mother centrioles re-gained centrobin during interphase? There is no comment about that, and statistics is not shown for Fig. 1 A.
The authors spent a substantial effort on complex treatments and analyses of mitotic cells to prove that centrobin removal is not required for distal appendage formation. However, if quantifications of centrosome phenotypes in Fig. 1A are accurate and if 25% of grandmother centrioles associate with centrobin, then it is self-evident that centrobin removal is not required for accumulation of distal appendage proteins. In addition, centrobin loss, per this quantification, occurs on some centrioles after several subdistal and distal appendage proteins had already localized to mother centrioles (for instance Odf2 and Cep83). These facts could be further reinforced by co-staining of centrobin and Cep164, SCLT1, and Cep83 in G2.
Point 3. Related to Point 2. The authors say that “in a normal metaphase cell OFD1 and Cep164 are present on both the grandmother and mother centriole”.
However, several publications have demonstrated that in cycling human prometaphase and metaphase cells, Cep164 is largely removed from grandmother centrioles and is not yet fully accumulated on mother centrioles (PMID: 30824690, PMID: 32211891). In addition, during mitotic arrest, Cep164 tends to re-accumulate on grandmother centrioles and accumulates on younger mother centrioles, as observed here by the authors (this reviewer has observed the same in multiple types of mitotic arrest).
Thus, the residual Cep164 signal in mitosis seems like an odd marker to use as a proxy for the presence of distal appendages. Some other distal appendage proteins, such as Cep83 or SCLT1, that associate with mother centrioles already in G2 and prophase would be more appropriate markers in this study.
Further, although Plk1 somehow transiently modulates Cep164 levels on centrioles upon mitotic entry (PMID: 30824690), Plk1 is not the only kinase that does that (PMID: 32211891, PMID: 30824690). So, clearly, there is a complex and still unexplored interplay between Plk1 and Cep164 localization in mitosis. This needs to be properly discussed in the manuscript and taken in consideration during data interpretation.
Point 4. Plk1 promotes centriole maturation and its long-term inhibition indirectly affects appendage formation. So, the data after 24h-long Plk1 inhibition, especially in combination with centrobin depletion (Fig. 5J and K) is ambiguous.

Point 5. The authors approach to the issue of centrosomal centrobin levels as if there were only two possibilities: present or absent. But there are intermediary levels of centrobin. I understand that it would be difficult to precisely measure the intensities of centrobin on individual centrioles, but some idea of how much of centrobin is lost and when during the cell cycle is needed.
Point 6. Based on the quantifications of centrobin on unduplicated mother centrioles, the authors suggest that centrobin is removed from mother centrioles because centrobin has the “higher affinity” for daughter centrioles. But they also argue that centrobin is actively removed in mitosis in a Plk1-dependent manner and by the presence of sub-distal appendage proteins. I am not sure how to fit these observations in the proposed concept. In addition, grandmother centrioles often do not have a “stock” of centrobin to be drawn from by daughter centrioles, meaning that centrobin is likely recruited from the cytoplasm and not from mother centrioles. Similarly, there is no direct evidence that Plk1 directly “transfers” centrobin from one centriole to another. This speculation seems to be adopted from the work in Drosophila neuroblasts (PMID: 32760088), it has not been investigated here, and I would strongly recommend it to be removed.
In Sas-6 depletion experiments, Sas6 signal needs to be shown in Figure 2 to attest the lack of daughter centrioles. Due to random orientation of centrioles Centrin-GFP signal may not be sufficient to judge centriole duplication status.

Point 7. It is unclear why siRNA was used to remove cenexin, since cenexin knockout cells were available. In addition, cenexin depletion by siRNA is incomplete (only ~50% for siRNA #1 and ~80% for siRNA#2, Fig. S2).
Point 8. It is unknown how the double depletion of cenexin and centrobin affects centriole structure, so this part of the data remains interpretable. The level of depletion has not been shown either.
Point 9. At the end of discussion, the authors speculate that timely removal of centrobin could affect the faithfulness of centriole duplication and cilia formation. But they don't provide any experiment or justification for this speculation. In fact, the data they show indicates that even without centrobin reduction, centrioles normally duplicate (Fig. 7).
Point 10. Writing.
In introduction: The statemet :"…they originalte as daughter centrioles...." is unclear.
In introduction: The authors describe centriole duplication as semi-conservative process (like DNA). However, mother centrioles do not split and do not serve as templates for daughter centriole assembly. In my view, this analogy is not useful.
In Introduction: Bowler at al., 2019 does not show that OFD1 is the first building step in the formation of distal appendages.
In Introduction: A description of centriole formation, maturation, and the timing of appendage formation needs to be reviewed to acutely reflect the current knowledge in the field.
In introduction, page 5, there is a statement that Plk1 kinase activity is required for loading of several distal appendage components such as Cep164.
However, Kong 2014 work does not claim that Plk1 directly loads appendage proteins onto centrosomes. In fact, acute Plk1 inhibition in interphase does not seem to affect their localization. The long-term effect of Plk1 inhibition on centriole maturation, opposed to the acute effects on localization of appendage proteins on already mature centrioles need to be clearly distinguished in writing.
On page 14, the authors claim that cenexin/Odf2, Cep128 and centriolin are present, in metaphase, only on grandmother centriole. However, previous studies have demonstrated that cenexin/ODF2 is abundantly present on both mother centrioles before and in mitosis (PMID: 30824690). The speculation that follows seems far-fetched.

Reviewer 3

Advance summary and potential significance to field

In this study Le Roux-Bourdieu and colleagues investigated the long standing question whether centrobin acts as a placeholder for distal appendage proteins until it is removed by the contribution of PLK1. They show that this is not the case and they show by codependency experiments and microscopy that the recruitment of key distal appendage proteins does not depend on centrobin removal. They come to the conclusion that PLK1 and sub-distal appendage proteins regulate the removal of centrobin and the build-up of distal appendages via separate pathways. I think they contribution has a significance especially in the field of distal appendage formation.

Comments for the author

(Since the manuscript text was not numbered, I will cite the criticized sections and write my comments below it) "To differentiate between the two possibilities, we looked at the cells depleted for SAS-6 for 24 hours, which in our experience can lead to the formation of 2:1 cells, in which only the grand-mother centriole gives rise to a daughter procentriole (Tan et al., 2015). The vast majority of 2:1 cells contained one centrobin-positive daughter centriole associated to the grandmother centriole, and no centrobin on the mother centriole (Fig 2E). We conclude that centrobin is removed from the mother centriole because daughter centrioles have a higher affinity for this protein."
> I think this is the weakest and least supported part of the manuscript.
Simply the presence of daughter centriole does not prove and existence of any kind of higher affinity towards centrobin. I would suggest to soften the expression to something like "daughter centrioles may have a higher affinity for this protein.

I suggest to test the centrobin localization upon STIL over-expression. In this case there are instances of a single mother with multiple daughter in a rosette formation (Arquint et al, 2012). I wonder how the distribution of centrobin varies between the daughters. I all of them share the centrobin pool (all of them have centrobin signal but weaker than a wild-type daughter) the higher affinity model could have some further support.
"To control whether centrobin presence at the mother centriole in 1:1 cells prevented this recruitment, we stained for OFD1: a large majority of 1:1 cells (75±2.5%) still displayed OFD1 at both grandmother and mother centrioles, even though the percentage was slightly lower than in 2:2 cells (91±1.3%; Fig. 3B and C)"
> Although there is a nice quantitation of Figure2 that the vast majority of mothers retain centrobin in 1:1 cells, it is more elegant (and to some extent essential) to show OFD1/CEP164 and centrobin co-staining on Fig3B/C.

First revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:

This is an interesting article on the dynamics of CNTROB and its role in distal appendages in human cell lines. It includes new data, which in general terms appears to be solid and reaches some interesting conclusions. These results merit publication once the following important points are addressed.

Reviewer 1 Comments for the Author:

1.- One main concern is about the major conclusion: the higher affinity of CNTRB for the newly born daughter centrioles is the cause of CNTRB removal from older centrioles. The authors themselves seem to be aware that this conclusion rests on thin evidence when in the introduction they write: “possibly because of higher affinity binding sites”. “Possibly” is the correct term here, but it is not used anywhere else in the manuscript. For instance in the abstract: “centrobin is removed from older centrioles “due to a higher affinity for the newly born daughter centrioles”; and in one heading of the results section: “Centrobin is removed from mother centriole due to a higher affinity for daughter centrioles”.

It is fairly obvious that proving this claim will take a great deal more than a series of immunofluorescence data. The provided data is fairly consistent with the authors’s interpretation, but does not falsify alternative hypotheses e.g daughter centrioles act catalytically. The authors must either provide additional data that demonstrate their conclusion or re-phrase the manuscript accordingly.

We agree with the reviewer that our conclusions were too strong, and we now state that our data are consistent with a model in which the daughter centrioles have a higher affinity, but we do not claim any more that our data demonstrate this model. As suggested by reviewer 3 we furthermore quantified centrobin levels in different centriole configurations. We found that in 1:1 cells there was twice as much centrobin on the mother centriole when compared to the wild-type daughter centrioles in 2:2 cells. In contrast, in spindle poles containing one grandmother or mother centriole and 2 centrioles, we found that centrobin levels on both daughter centrioles reached 60%. Both results thus provide further support an affinity model (see new Figure 2). Note that to achieve the latter configuration, we treated RPE1 cells with low doses of the Plk4 inhibitor centrinone. This led to centriole over-duplication, similar to what has been observed after overexpression of catalytically-inactive Plk4 (Guderian et al., 2010), most likely because the trans- phosphorylation dependent Plk4 degradation is blocked without yet interfering with the ability of Plk4 to initiate centriole duplication.

2.- In Figure 2, CNTROB signal intensity is much lower in the mother centriole of the PLK4i-treated cell than in the daughter centriole of the control cell. This result is not consistent

with the hypothetical mechanism proposed in the manuscript. If CNTROB removal was due to a higher affinity for daughter centrioles, CNTROB signal intensity should be as high in PLK4i-treated than in control cells. Quantitated data on CNTROB signal intensity in these centrioles are in order.

First, we emphasize that the images shown in the original Figure 2 assessed centrobin localization in a qualitative manner (present or not present) only and that the intensities between the 1:1 and 2: 2 cells were not necessarily comparable. Nevertheless, as suggested by the reviewer we now performed a quantitative analysis (see also point 1), showing that mother centrioles in 1:1 cells have on average twice as much centrobin as daughter centrioles in 1:1 cells (see new Figure 2).

3.- The point that CNTROB localisation is compatible with OFD1 and CEP164 is important, but Figure 3B,E does not serve the purpose because it does not show CNTROB. Bearing in mind Figure 2 one can guess the result, but co-staining for CNTROB is a must to confirm its presence on these centrioles. This is even more important when taking into account Wang et al., 2018 showing that PACT-CNTROB, which forces CNTROB centriolar localisation through the cell cycle, results in the loss of appendages.

We agree with the reviewer and provide now images of 1:1 cells that were co-stained for ODF1 and CEP164 and Centrobin, confirming our initial results (see Figure 2F and 3B).

4.- Another major issue that requires attention derives from drawing general conclusions out of specific observations. Results from the only one animal in which Centrobin has been studied in vivo demonstrate that Centrobin is a multifaceted protein that can exert different, sometimes seemingly contradictory functions. Thus, for instance Centrobin promotes PCM recruitment during interphase in Drosophila neuroblasts, but not in other cell types like epithelial wing disc cells. Likewise Centrobin inhibits centriole- to-basal body conversion in type I neurons (Gottardo et al., 2015 DOI: 10.1016/j.cub.2015.07.038) but is required for proper basal body function in spermatocytes (Reina et al., 2018 DOI: 10.1083/jcb.201801032). Incidentally, Centrobin's function inhibiting cilium growth in Drosophila neurons could be mentioned in the manuscript.

Statements assuming that observations made on a handful of human cell lines can be generalized to “mammalian cells” are likely to be proven wrong once in vivo studies allow for Centrobin dynamics and function to be studied in different cell types in a living mammalian experimental model. In Drosophila, as explained before, statements generalising Centrobin function are definitively wrong and so are general statements on Centrobin localisation which is daughter centriole-specific in many somatic cells and early stage germline cells, but localises to both mother and daughter centrioles in primary spermatocytes (Reina et al., 2018).

Throughout the manuscript, conclusions should refer to the cell type in which the observation has been made, hence avoiding unwarranted generalisations. This applies both to the new data reported in the manuscript as well as to references to published data.

We thank the reviewer for these insightful comments. We now include in the introduction the fact that Centrobin function can vary from cell to cell, and we specify in our conclusions and discussion that our results are valid for human epithelial cells. We also more explicitly point out that we are using the same cell line as the Wang et al., study that proposed the centrobin placeholder theory.

5.-Being so heavily based on immunofluorescence, it would be advisable to back up at least the conclusions derived from Figure 1 with a second anti-CNTROB antibody (if I am not mistaken, the entire manuscript relies on the Abcam anti-CNTROB).

We agree with the reviewer and have now quantified centrobin levels on the different centrioles with a second, independent centrobin antibody from Sigma, which confirmed our initial characterization (see supplementary Fig. 1)

6.- There are recurrent mistakes regarding the description of Centrobin function and localisation in Drosophila that must be amended:

6a.- “In Drosophila neuroblasts, which contain appendage-free centrioles, centrobin is exclusively present on the younger centrosome containing the mother centriole, whose identity it controls (Januschke et al., 2013)”

6b.-“note that in flies centrobin is present not on daughter centrioles but on the younger centrosome (Januschke et al., 2013)”

6c.- “In Drosophila, centrobin localization has to be controlled since it regulates the fate of the young centrosome”, enabling it to organize microtubules and to be retained by the stem cell during asymmetric cell division (Januschke et al., 2013).”

The authors got Centrobin localisation in Drosophila neuroblasts wrong. In these cells, CNB is only present in the DAUGHTER centriole, not in the mother. And the daughter centriole is in the younger centrosome.

Once more, general statements like “note that in flies” or “In Drosophila” are bound to be wrong, and so are these ones. Centrobin is daughter centriole-specific in neuroblasts and male germline stem cells, for instance, but it is not daughter centriole-specific in primary spermatocytes.

Moreover, Centrobin effect on the young centrosome’s fate is only true in neuroblasts. It is not true in other Drosophila cells including stem cells like those of the male germline.
We thank the reviewer for pointing out these factual errors, which we now have corrected by being more precise on the cell type these observations were based on, and by specifying at which cell cycle stage we are referring to a mother or daughter centriole.

Other points

The results regarding the effect of Cenexin, centriolin and CEP128 depletion in mitosis are clear, but what happens to CNTROB localisation in G1?
As suggested by the reviewer, we have analyzed the centrobin pattern in G1 knock-out cenexin, centriolin or CEP128 cells, and found that centrobin removal from the mature centriole was severely impaired in cenexin knock-outs and somewhat impaired in centriolin and CEP128 KO cells, confirming the involvement of subdistal appendage proteins in this process (see new Supplementary Figure 4A).

Figure 4h, Aurora-Ai: the two middle panels are swapped.

This point has been corrected. We thank the reviewer

Figure 7b: Is in unclear which panels belong to each genotype.

This point has been corrected. We thank the reviewer

The article demonstrating that in Drosophila neuroblasts Centrobin is daughter centriole-bound and that these cells retain the younger (daughter) centrosome is Januschke et al., 2011 https://doi.org/10.1038/ncomms1245

This point has been corrected. We thank the reviewer

Reviewer 2 Advance Summary and Potential Significance to Field:

Centrosomes are organelles built of centrioles, cylindrical MT-based structures, that, when fully assembled carry subdistal and distal appendages, responsible for MT anchoring, and ciliation, respectively. Centrobin is a centrosomal protein enriched to the sites of developing centrioles from their earliest stages. During centriole development, centrobin levels are reduced. This reduction has been suggested to be a prerequisite for the formation of centriole appendages. In this work, Le Roux-Bourdieu and colleagues explore the timing and the consequences of centrobin removal from mother centrioles, and the role of Plk1 and subdistal appendage proteins in this process.

They find that centrobin is lost from younger mother centrioles during their second mitosis. They show that distal appendage proteins re recruited to mothercentrioles independently of centrobin removal. They further demonstrate that Plk1 controls centrobin localization at centrosomes, but independently from distal appendage proteins. Finally, using knockouts for subdistal appendage proteins cenexin/Odf2, Centriolin and Cep128, they show that removal of these proteins leads to retention of some centrobin on mother centrioles.

There are interesting findings in the manuscript. For instance, the timeline of centrobin removal is described in more detail than before. They clarify that localization of Cep164 does not require removal of centrobin from mother centrioles. The authors show that Plk1 activity can modulate centrosomal levels of centrobin.

However, there are issues with data interpretation, proposed concepts, and image quality. Nevertheless, the subject is important and interesting, and the study could be publishable after an extensive revision.

Reviewer 2 Comments for the Author:

Point 1. Immunofluorescence signals are hard to analyze because they are distorted. Specifically, they are elongated diagonally, always in same direction, which indicates some systemic issue with imaging. Because of that, it remains unclear how accurate are the quantifications of centrosome phenotypes and centrobin IF signals. At a minimum, the data in 1D, 2A, 2D, 3B, 5G, 7B, and 7F needs to be replaced with properly acquired, high-quality images.

We thank the reviewer for the comment and have now included images which we hope are of sufficient quality in Figure 1, 2, 3 and 7.

Point 2. Per quantification in (Fig. 1A), centrobin is associated with both centrioles in ~10% of G1 cells. However, the number of cells with both mother centrioles associating with centrobin increases in S and G2 to 15% and >25% respectively. Have some older mother centrioles re-gained centrobin during interphase? There is no comment about that, and statistics is not shown for Fig.1 A.
The percentage of centrobin-positive grandmother centriole does not vary significantly in G1, S and G2, but we agree with the reviewer that it is better to state this explicitly, which we now have done. We also now complement in Figure 1 our qualitative analysis with a quantitative analysis of Centrobin levels per centriole at the different cell cycle stages.

The authors spent a substantial effort on complex treatments and analyses of mitotic cells to prove that centrobin removal is not required for distal appendage formation. However, if quantifications of centrosome phenotypes in Fig. 1A are accurate and if 25% of grandmother centrioles associate with centrobin, then it is self-evident that centrobin removal is not required for accumulation of distal appendage proteins. In addition, centrobin loss, per this quantification, occurs on some centrioles after several subdistal and distal appendage proteins had already localized to mother centrioles (for instance Odf2 and Cep83). These facts could be further reinforced by co-staining of centrobin and Cep164, SCLT1, and Cep83 in G2.

We agree that the presence of centrobin on a minority grandmother centrioles speaks against the requirement of centrobin removal for distal appendage formation; nevertheless we believe that the different experimental conditions presented in this study are necessary to dis-prove the centrobin placeholder hypothesis proposed by Wang et al., 2018. We also agree that presenting co-staining of Cep164 and Centrobin in G2 cells does re-inforce that message, which is why we had added just such images in the original Figure. 3F. Nevertheless to reinforce this message we now also provide new images of G2 cells co-stained with centrobin and OFD1, the protein that is initially recruited for the formation of distal appendages in the new figure 3G.

Point 3. Related to Point 2. The authors say that “in a normal metaphase cell, OFD1 and Cep164 are present on both the grandmother and mother centriole”. However, several publications have demonstrated that in cycling human prometaphase and metaphase cells, Cep164 is largely removed from grandmother centrioles and is not yet fully accumulated on mother centrioles (PMID: 30824690, PMID: 32211891). In addition, during mitotic arrest, Cep164 tends to re-accumulate on

grandmother centrioles and accumulates on younger mother centrioles, as observed here by the authors (this reviewer has observed the same in multiple types of mitotic arrest). Thus, the residual Cep164 signal in mitosis seems like an odd marker to use as a proxy for the presence of distal appendages. Some other distal appendage proteins, such as Cep83 or SCLT1, that associate with mother centrioles already in G2 and prophase would be more appropriate markers in this study. Further, although Plk1 somehow transiently modulates Cep164 levels on centrioles upon mitotic entry (PMID: 30824690), Plk1 is not the only kinase that does that (PMID: 32211891, PMID: 30824690). So, clearly, there is a complex and still unexplored interplay between Plk1 and Cep164 localization in mitosis. This needs to be properly discussed in the manuscript and taken in consideration during data interpretation.

Although we agree that CEP164 are reduced on metaphase centrosomes, we would like to point out that Kong et al., JCB 2014 (Figure 4) shows that in metaphase RPE1 cells, CEP164 is equally present on both grandmother and mother centrioles, at 25-30% when compared to maximal levels in S-phase. In the study cited by the reviewer (Bowler et al., Nat Comm, 2019) CEP164 is clearly present on both mother and grandmother centrioles in metaphase (Fig. 5d). Finally, our own data show that in unperturbed metaphase RPE1 cells, CEP164 is present at both grandmother and mother centrioles (see e.g. new Figure 3G). We therefore stand by our statement, which we think is factually correct. Also, apart from the STLC-treated cells, all the mitotic cells shown in this study are examples of unsynchronized cells that were not specifically arrested in mitosis, therefore our staining does not represent a synchronization artifact. Finally, as stated in our introduction we used CEP164 as a proxy, as the study presenting the centrobin placeholder hypothesis (Wang et al., 2018), showed that in the dependency chain CEP164 is the last protein recruited to distal appendages. While we agree that one could repeat all the study with alternative antibodies such as CEP83, this would require a very large amount of additional work that would go beyond the frame for a major revision (in preliminary experiments we tested CEP83 antibodies on mitotic cells, and found a signal to noise ratio in terms of centrosome signal that was not better than the one with CEP164 antibodies).

We nevertheless agree that the regulation of CEP164 at the different centrioles is complex, and that our initial manuscript was not precise enough. We now specify throughout our text that we used the recruitment of CEP164 at the mother centriole in metaphase as a readout for distal appendage protein recruitment (and not distal appendage formation) at this specific stage and on this specific centriole, but also point out in the discussion that other mitotic kinases regulate CEP164 localization to avoid giving the impression that Plk1 is the sole regulator of this process.

Point 4. Plk1 promotes centriole maturation and its long-term inhibition indirectly affects appendage formation. So, the data after 24h-long Plk1 inhibition, especially in combination with centrobin depletion (Fig. 5J and K) is ambiguous.

The aim of Figure 5 is to show that Plk1 regulates CEP164 localization independently of centrobin localization. The first set of data, i.e. that the long term inhibition of Plk1 reduces the recruitment of CEP164 at the mother centriole, most likely by preventing centriole maturation, has already been described (Kong et al., 2014), and is therefore not the point of this experiment. Our point is to prove that this effect does not occur via centrobin, i.e. to show that we can functionally separate the regulation of CEP164 localization by Plk1 from the regulation of centrobin localization by Plk1. We believe that this is the case, as the reduced levels of CEP164 in Plk1-inhibited cells did not depend on centrobin.

Point 5. The authors approach to the issue of centrosomal centrobin levels as if there were only two possibilities: present or absent. But there are intermediary levels of centrobin. I understand that it would be difficult to precisely measure the intensities of centrobin on individual centrioles, but some idea of how much of centrobin is lost and when during the cell cycle is needed.

We thank the reviewer for this suggestion, and have now in the new Figure 1 complemented our qualitative analysis (present or not present), with a quantitative analysis of centrobin-levels per centriole, which together better reflect the dynamic nature of centrobin removal during the cell cycle.

Point 6. Based on the quantifications of centrobin on unduplicated mother centrioles, the authors suggest that centrobin is removed from mother centrioles because centrobin has the “higher affinity” for daughter centrioles. But they also argue that centrobin is actively removed in mitosis in a Plk1-dependent manner and by the presence of sub-distal appendage proteins. I am not sure how to fit these observations in the proposed concept. In addition, grandmother centrioles often do not have a “stock” of centrobin to be drawn from by daughter centrioles, meaning that centrobin is likely recruited from the cytoplasm and not from mother centrioles. Similarly, there is no direct evidence that Plk1 directly “transfers” centrobin from one centriole to another. This speculation seems to be adopted from the work in Drosophila neuroblasts (PMID: 32760088), it has not been investigated here, and I would strongly recommend it to be removed.

In accordance with similar comments with reviewer 1 and 3, we now explicitly state that our results do not “prove” the high affinity model, but state that our data are compatible with such a model, particularly in light of the new quantification experiments we performed in 1:1, 2:2 and the new 3:3 cells, showing that centrobin on individual centrioles in mitosis are inversely proportional to the number of “youngest” centrioles. We agree that experimentally proving this hypothesis would require biochemical experiments, but we believe that our results are sufficiently strong to propose such a model and state that our data are compatible with such an interpretation.

In Sas-6 depletion experiments, Sas6 signal needs to be shown in Figure 2 to attest the lack of daughter centrioles. Due to random orientation of centrioles, Centrin-GFP signal may not be sufficient to judge centriole duplication status.

We now include in the supplementary Figure S2 immunofluorescence pictures demonstrating that Sas-6 siRNA is efficient and sufficient to prevent centriole duplication.

Point 7. It is unclear why siRNA was used to remove cenexin, since cenexin knockout cells were available. In addition, cenexin depletion by siRNA is incomplete (only ~50% for siRNA #1 and ~80% for siRNA#2, Fig. S2).

In the course of this study, we first performed siRNA experiments with two different set of siRNAs, which were consistent, before studying cenexin knock-out cells, which gave a similar, yet weaker phenotype. As we now state explicitly in the discussion we interpret this qualitative difference as the result of an adaption of knock-out cells, which is known to occur. (Rossi et al., 2015, Nature). Since we already had obtained the full set of results, we fell that it was important for the readership to have access to all these results, and our corresponding speculative interpretation, with which they can agree or not.

Point 8. It is unknown how the double depletion of cenexin and centrobin affects centriole structure, so this part of the data remains interpretable. The level of depletion has not been shown either.

We respectfully disagree, the depletion levels of each protein in the co-depletion was shown in in the original supplementary Figure 2E-H (now Fig. 3E-H), confirming that our double depletion was as effective as the depletion of the single protein.

Point 9. At the end of discussion, the authors speculate that timely removal of centrobin could affect the faithfulness of centriole duplication and cilia formation. But they don’t provide any experiment or justification for this speculation. In fact, the data they show indicates that even without centrobin reduction, centrioles normally duplicate (Fig. 7).

We partially disagree, as the final sentence of our discussion contained a relative sentence in which we cited two experimental studies showing that overexpression of centrobin can affect the robustness of centriole elongation and the structure of the axoneme in the cilia. We therefore feel that our speculation is based on experimental data. Nevertheless, to be more specific, we now explicitly state which specific step of the centrosome duplication cycle might be affected (centriole elongation).

Point 10. Writing. In introduction: The statemet :"…they originalte as daughter centrioles...." is unclear.

We replaced “originate” with “emerge”, which is more precise.

In introduction: The authors describe centriole duplication as semi-conservative process (like DNA). However, mother centrioles do not split and do not serve as templates for daughter centriole assembly. In my view, this analogy is not useful.

Here we respectfully disagree. We wrote that CENTROSOME duplication was semi-conservative (not CENTRIOLE duplication). In G1 the two centrioles of a centrosome dis-engage (equivalent to splitting), and each centriole (equivalent to a DNA strand) serves as a template for the formation of a daughter centriole. So while we agree that Centriole duplication is conservative, CENTROSOME duplication is semi- conservative, as originally demonstrated by Kochanski and Borisy, JCB, 1990.

In Introduction: Bowler at al., 2019 does not show that OFD1 is the first building step in the formation of distal appendages.

We agree and have corrected this point in the text

In Introduction: A description of centriole formation, maturation, and the timing of appendage formation needs to be reviewed to acutely reflect the current knowledge in the field.

We now have included in the introduction a more precise description of the process, specifying that mother centrioles recruit distal and subdistal appendage proteins as they progress through mitosis to drive the formation of the respective appendages in the next G1.

In introduction, page 5, there is a statement that Plk1 kinase activity is required for loading of several distal appendage components such as Cep164. However, Kong 2014 work does not claim that Plk1 directly loads appendage proteins onto centrosomes. In fact, acute Plk1 inhibition in interphase does not seem to affect their localization. The long-term effect of Plk1 inhibition on centriole maturation, opposed to the acute effects on localization of appendage proteins on already mature centrioles need to be clearly distinguished in writing.

First, we did not claim in our introduction that Plk1 directly loads appendage proteins onto centrosomes, we claim based on the Kong et al., study that Plk1 activity is required. Whether it is direct or indirect cannot be evaluated at this stage, which we now state explicitly. Second, we don’t think that an acute effect of Plk1 can excluded based on the Kong et al. 2014 data. Looking at Figure 5d, it is evident that already an acute inhibition of Plk1 (2h) can lead to a strong reduction (-40%) in the proportion of cells bearing CEP164 signals on both mother and grandmother centrioles in interphase. Nevertheless, to be more precise we now talk throughout our text of distal appendage protein recruitment on the mother centriole, and we state that Plk1 activity enables the recruitment of distal appendage proteins, possibly by promoting centriole maturation.

On page 14, the authors claim that cenexin/Odf2, Cep128 and centriolin are present, in metaphase, only on grandmother centriole. However, previous studies have demonstrated that cenexin/ODF2 is abundantly present on both mother centrioles before and in mitosis (PMID: 30824690). The speculation that follows seems far-fetched.

The reviewer is correct that we should not use “exclusively on the grandmother centriole”, but it would be incorrect to state that Odf2 is present on both mother and grandmother centrioles in equal amounts. Indeed Kong et al. 2014 and Bowler et al. 2019 in the Loncarek laboratory, our laboratory in 2015 (Gasic et al., 2015), the Hehnly laboratory (Colicino et al., 2019) or the Doxsey laboratory (Hung et al., 2016) report that Cenexin is predominantly or sometimes only present on the grandmother centriole (5-fold enrichment reported in the Bowler et al, 2019 study cited by the reviewer). Therefore, we changed to “predominantly” instead of exclusively.

Reviewer 3 Advance Summary and Potential Significance to Field:

In this study Le Roux-Bourdieu and colleagues investigated the long standing question whether centrobin acts as a placeholder for distal appendage proteins until it is removed by the contribution of PLK1. They show that this is not the case and they show by codependency experiments and microscopy that the recruitment of key distal appendage proteins does not

depend on centrobin removal. They come to the conclusion that PLK1 and sub-distal appendage proteins regulate the removal of centrobin and the build- up of distal appendages via separate pathways. I think they contribution has a significance especially in the field of distal appendage formation.

Reviewer 3 Comments for the Author:(Since the manuscript text was not numbered, I will cite the criticized sections and write my comments below it)

We thank the reviewer for the supportive comments, and have addressed his/her concerns in the following manner:

"To differentiate between the two possibilities,we looked at the cells depleted for SAS-6 for 24 hours, which in our experience can lead to the formation of 2:1 cells, in which only the grand-mother centriole gives rise to a daughter procentriole (Tan et al., 2015). The vast majority of 2:1 cells contained one centrobin-positive daughter centriole assßociated to the grandmother centriole, and no centrobin on the mother centriole (Fig 2E). We conclude that centrobin is removed from the mother centriole because daughter centrioles have a higher affinity for this protein."

> I think this is the weakest and least supported part of the manuscript. Simply the presence of daughter centriole does not prove and existence of any kind of higher affinity towards centrobin. I would suggest to soften the expression to something like "daughter centrioles may have a higher affinity for this protein. I suggest to test the centrobin localization upon STIL over-expression. In this case there are instances of a single mother with multiple daughter in a rosette formation (Arquint et al, 2012). I wonder how the distribution of centrobin varies between the daughters. I all of them share the centrobin pool (all of them have centrobin signal but weaker than a wild-type daughter) the higher affinity model could have some further support.

We agree with the reviewer that our conclusions were too strong, and we now state that our data are consistent with a model in which the daughter centrioles have a higher affinity, but we do not claim any more that our data demonstrate this model. As suggested by the reviewer we quantified centrobin levels in different centriole configurations (new Figure 2). We found that in 1:1 cells there was twice as much centrobin on the mother centriole when compared to the wild-type daughter centrioles in 2:2 cells. In contrast, in spindle poles containing one grandmother or mother centriole and 2 centrioles, we found that centrobin levels on both daughter centrioles reached 60%. Both results thus provide further support an affinity model. Note that to achieve the latter configuration, we treated RPE1 cells with low doses (25nM) of the Plk4 inhibitor centrinone. This led to centriole over-duplication, similar to what has been observed after overexpression of catalytically-inactive Plk4 (Guderian et al., 2010), most likely because the trans- phosphorylation dependent Plk4 degradation is blocked without yet interfering with the ability of Plk4 to initiate centriole duplication.

"To control whether centrobin presence at the mother centriole in 1:1 cells prevented this recruitment, we stained for OFD1: a large majority of 1:1cells (75±2.5%) still displayed OFD1 at both grandmother and mother centrioles, even though the percentage was slightly lower than in 2:2 cells (91±1.3%; Fig. 3B and C)"
> Although there is a nice quantitation of Figure2 that the vast majority of mothers retain centrobin in 1:1 cells, it is more elegant (and to some extent essential) to show OFD1/CEP164 and centrobin co-staining on Fig3B/C.

We agree with the reviewer and provide the co-staining in the new figures 2F and 3B.

Second decision letter

MS ID#: JOCES/2021/259120

MS TITLE: PLK1 controls centriole distal appendage formation and centrobin removal via independent pathways

AUTHORS: Morgan LeRoux-Bourdieu, Devashish Dwivedi, Daniela Harry, and Patrick Meraldi
ARTICLE TYPE: Research Article

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers gave favourable reports but raised some critical points that will require amendments to your manuscript. I hope that you will be able to carry these out because I would like to be able to accept your paper, depending on further comments from reviewers.

We are aware that you may be experiencing disruption to the normal running of your lab that makes experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion.

I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript presents evidence that substantiates a key role for Plk1 kinase in regulating centrobin removal and distal appendage formation during centriole maturation.

Comments for the author

The authors have addressed all the points that I raised. I recommend publication.

Reviewer 2

Advance summary and potential significance to field

The authors have put a significant effort to answer reviewers’ questions and the manuscript has been improved.

Comments for the author

The authors provide additional evidence to attest that centrobin is repositioned from mother centriole to procentrioles due to their higher affinity to centrobin. They utilize Plk4 inhibitor to

generate mother centrioles associated with two procentrioles instead of one. They measure the levels of centrobin on such procentrioles and find that they are variable and lower than in control procentrioles. The authors use this result to strengthen the argument that centrobin is gradually removed from mother centrioles to procentrioles due to their high affinity for centrobin. However, these results are ambiguous and proposed idea remains unproven.
Under conditions the authors describe, it is not surprising to find procentrioles of various stages of assembly and, hence, various levels of centrobin. The reason likely is that procentrioles can initiate asynchronously at various parts of the cell cycle, if Plk4 levels are manipulated. More sophisticated analysis, including the analysis of procentriole structure, would be needed to interpret this data and reach the conclusion.
Accidentally, I have a fair knowledge of centriolar rosettes (mother centrioles associated with multiple procentrioles). In addition, centrobin is frequently used in my lab to mark procentrioles (both in diplosomes and in rosettes). In our hands, in rosettes, cumulative levels of procentriole-associated centrobin exceed the levels of mother centriole-associated centrobin at the beginning of the S phase multiple times. Thus, this extra centrobin must be recruited from the cytoplasm. At the same time, mother centriole centrobin does not seem to be exhausted during procentriole formation.
Finally, the fact that procentriole(s) that are formed in association with centrobin-negative grandmothers normally recruit centrobin also strongly argues against authors' concept.
I would strongly recommend that, if accepted, the manuscript does not include the speculation of the affinity based centrobin re-distribution to procentrioles and the set of data related to it. A simple explanation would be more appropriate. For instance: mother centrioles could lose centrobin-binding epitopes during maturation.
The authors suggest that Plk1 inhibition leads to re-binding of centrobin to older centrioles. However, centrobin can associates with PCM components (for instance in Drosophila neuroblasts it associates with PCM components). Due to low imaging resolution, it is not clear whether observed re-associated centrobin localizes to the same place from which it had been lost. This needs to be clarified in the text, as the reader automatically assumes that centrobin is re-loaded to centriole distal ends. This may not be the case.
Wording that needs to be corrected:
In the abstract: "Centrioles emerge (or originate, in the original version) as daughter centrioles from existing centrioles". This suggests that procentrioles they form directly from existing centrioles in S-phase. Similarly, in the results, line 22 says that procentriole arise from mother centrioles. They are assembled adjacent to the mother centriole, but they don't arise from them.
"[centrioles]…reach their full functionality with the formation of distal and subdistal appendages two mitosis later". This could be misunderstood as if there are functional subdistal or distal appendages present in mitosis.
The authors say that centrobin is "needed for centrosome duplication". However using authors' definition from rebuttal, centrosome duplication is semiconservative and involves splitting of one centrosome in two due to centriole separation (disengagement). So, centrobin is not needed for centrosome duplication, since centrioles have not been shown not to fail disengagement in the absence of centrobin. I believe the authors meant to say that centrobin is important for centriole assembly?
The work in neuroblast (Januschke et al., 2013) does not seem to show the "transfer" of centrobin from the mother centriole to the daughter centriole, as the authors state in discussion.

Reviewer 3

Advance summary and potential significance to field

In this study Le Roux-Bourdieu and colleagues investigated the long-standing question whether centrobin acts as a placeholder for distal appendage proteins until it is removed by the contribution of PLK1. They show that this is not the case and they show by codependency experiments and microscopy that the recruitment of key distal appendage proteins does not depend on centrobin removal. They come to the conclusion that PLK1 and sub-distal appendage proteins regulate the removal of centrobin and the build-up of distal appendages via separate pathways. I think they contribution has a significance especially in the field of distal appendage formation. All the substantial comments were addressed thus I think is it suitable for publication.
Comments for the author

In this new version of the manuscript, the authors addressed my previous comments sufficiently thus I recommend this paper for publication.

Second revision

Author response to reviewers' comments

Reviewer 1 Comments to the Author:
The authors have addressed all the points that I raised. I recommend publication.

Reviewer 3 Comments to the Author:
In this new version of the manuscript, the authors addressed my previous comments sufficiently thus I recommend this paper for publication.
We thank both reviewers for their supportive comments

Reviewer 2 Comments to the Author:
1. The authors provide additional evidence to attest that centrobin is repositioned from mother centriole to procentrioles due to their higher affinity to centrobin. They utilize Plk4 inhibitor to generate mother centrioles associated with two procentrioles instead of one. They measure the levels of centrobin on such procentrioles and find that they are variable and lower than in control procentrioles. The authors use this result to strengthen the argument that centrobin is gradually removed from mother centrioles to procentrioles due to their high affinity for centrobin. However, these results are ambiguous and proposed idea remains unproven. Under conditions the authors describe, it is not surprising to find procentrioles of various stages of assembly and, hence, various levels of centrobin. The reason likely is that procentrioles can initiate asynchronously,at various parts of the cell cycle, if Plk4 levels are manipulated. More sophisticated analysis, including the analysis of procentriole structure, would be needed to interpret this data and reach the conclusion. Accidentally, I have a fair knowledge of centriolar rosettes (mother centrioles associated with multiple procentrioles). In addition, centrobin is frequently used in my lab to mark procentrioles (both in diplosomes and in rosettes). In our hands, in rosettes, cumulative levels of procentriole-associated centrobin exceed the levels of mother centriole-associated centrobin at the beginning of the S phase multiple times. Thus, this extra centrobin must be recruited from the cytoplasm. At the same time, mother centriole centrobin does not seem to be exhausted during procentriole formation. Finally, the fact that procentriole(s) that are formed in association with centrobin-negative grandmothers normally recruit centrobin also strongly argues against authors' concept.

I would strongly recommend that, if accepted, the manuscript does not include the speculation of the affinity based centrobin re-distribution to procentrioles and the set of data related to it. A simple explanation would be more appropriate. For instance: mother centrioles could lose centrobin-binding epitopes during maturation.

We thank the reviewer for these detailed comments. For the following reasons, we would like to retain the affinity hypothesis, albeit in a more precise form that is very close to the suggestion of the reviewer:

1. First, we can only interpret our results based on our own results and the published data, and it is not possible to take in account unpublished data of the reviewer that we cannot see ourselves. The data obtained by the reviewer could be in a different cell line, or the situation in which the reviewer monitors the formation of rosettes could be very different, i.e. over a longer time period, during which new centrobin is synthesized in the cell, versus a short time window (prometaphase), during which centrobin leaves the mother centriole and re-appears on the daughter centrioles, while very little transcription/translation is going on. We also emphasize that throughout our manuscript we present this hypothesis explicitly as a speculative hypothesis that needs to be proven later, leaving the reader the possibility to draw his/her own conclusions. The reviewers asked us for additional

supportive experiments, which provided results that were still consistent with our model, and we therefore believe that our discussion can include such a speculation.

2. A second, even more important point is that we believe that there is a misunderstanding. Our affinity model did not mean to infer that centrobin is directly stripped from mother centrioles for a direct transfer to daughter centrioles, as the reviewer concludes "*Finally, the fact that procentriole(s) that are formed in association with centrobin-negative grandmothers normally recruit centrobin also strongly argues against authors' concept*". Instead, as we know explicitly state in our discussion, our model infers a dynamic centrobin exchange between mother centrioles, the cytoplasm, and daughter centrioles. As centrobin in our experimental conditions is present in limited amounts it will accumulate on the higher-affinity sites of the daughter centrioles and fail to remain on the mother centrioles. In fact, when the reviewer proposes a "*simple, more appropriate explanation*" he/she invokes affinity-base mechanism "mother centrioles could lose centrobin-binding epitopes during maturation". The only difference is that we postulate that mother centrioles do not completely lose the ability to bind centrobin, it is just that the centrobin-binding epitopes have a weaker affinity than those present on the daughter centrioles. Our model thus explains why mother centrioles retain centrobin in 1:1 cells, whilst the simple explanation of reviewer 2 is not compatible with those data set.

2. The authors suggest that Plk1 inhibition leads to re-binding of centrobin to older centrioles. However, centrobin can associates with PCM components (for instance in Drosophila neuroblasts it associates with PCM components). Due to low imaging resolution, it is not clear whether observed re-associated centrobin localizes to the same place from which it had been lost. This needs to be clarified in the text, as the reader automatically assumes that centrobin is re-loaded to centriole distal ends. This may not be the case.
We have taken this comment in account, adding in the discussion: "*Our present resolution also does not allow us to distinguish whether in Plk1 inhibited cells, centrobin re-associates to the same distal end of mother centrioles as control S-phase cells.*" Nevertheless, we emphasize that centrobin re-localizes to the mother centriole, not the PCM, as the localization pattern is very different from the one we observed with antibodies against gamma-tubulin or pericentrin, two typical PCM markers.

3. In the abstract: "Centrioles emerge (or originate, in the original version) as daughter centrioles from existing centrioles". This suggests that procentrioles they form directly from existing centrioles in S-phase. Similarly, in the results, line 22 says that procentriole arise from mother centrioles. They are assembled adjacent to the mother centriole, but they don't arise from them.
"[centrioles]…reach their full functionality with the formation of distal and subdistal appendages two mitosis later". This could be misunderstood as if there are functional subdistal or distal appendages present in mitosis.
We now wrote in the abstract "*In human cells daughter centrioles are assembled adjacent to existing centrioles in S-phase and reach their full functionality with the formation of distal and subdistal appendages one-and-a-half cell cycle later, as they exit their second mitosis.*"

4. The authors say that centrobin is "needed for centrosome duplication". However, using authors' definition from rebuttal, centrosome duplication is semiconservative and involves splitting of one centrosome in two due to centriole separation (disengagement). So, centrobin is not needed for centrosome duplication, since centrioles have not been shown not to fail disengagement in the absence of centrobin. I believe the authors meant to say that centrobin is important for centriole assembly?
We had specifically written that centrobin is required for **efficient** centrosome duplication, but we now explicitly state : it is required for efficient centriole elongation during centrosome duplication"

5. The work in neuroblast (Januschke et al., 2013) does not seem to show the "transfer" of centrobin from the mother centriole to the daughter centriole, as the authors state in discussion.
We now have removed this reference at this point of the discussion.

Third decision letter

MS ID#: JOCES/2021/259120

MS TITLE: PLK1 controls centriole distal appendage formation and centrobin removal via independent pathways

AUTHORS: Morgan LeRoux-Bourdieu, Devashish Dwivedi, Daniela Harry, and Patrick Meraldi
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.